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THE USE OF DNA FLOW CYTOMETRY AS A PROGNOSTIC INDICATOR AFTER LOCAL RECURRENCE IN CONSERVATIVELY TREATED CLINICAL STAGE I AND II BREAST CANCERS

Margaret M. Toth

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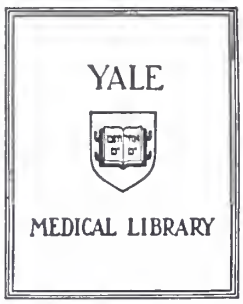
1991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THE USE OF DNA FLOW CYTOMETRY AS A PROGNOSTIC INDICATOR AFTER LOCAL RECURRENCE IN CONSERVATIVELY TREATED CLINICAL STAGE I AND II BREAST CANCERS

A Thesis Submitted to the Yale University School of Medicine in Partial Fullfillment of the Requirements for the Degree of Doctor of Medicine

by

Margaret M. Toth

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THE USE OF DNA FLOW CYTOMETRY AS A PROGNOSTIC INDICATOR AFTER LOCAL RECURRENCE IN CONSERVATIVELY TREATED CLINICAL STAGE I AND II BREAST CANCERS. Margaret M. Toth, Stuart D. Flynn, and Bruce G. Haffty. Department of Therapeutic Radiology, Yale University, School of Medicine, New Haven, CT.

Abstract

Over the past decade, breast conserving surgery with primary radiotherapy has been frequently employed as an option for the treatment of early breast cancer. With increasing follow-up, it is clear conservatively treated patients have a long-term risk for local recurrence. To date, prognostic indicators following recurrence are limited to clinical and pathological observations. No objective prognostic parameters have been established.

The purpose of the research summarized in this thesis was to determine whether DNA flow cytometry is a useful indicator of prognosis in patients who have suffered local recurrence following conservative treatment. Archival breast tissue specimens from tumor recurrences were obtained from 38 of the 50 women in the Yale-New Haven Hospital series who suffered local failure following conservative treatment. Median follow-up after recurrence was 5.06 years.

DNA flow cytometric measurements of DNA-ploidy and S-phase fraction were performed on all available samples. 62% of patients had DNA-diploid recurrent tumors. DNA-ploidy was predictive of both overall and disease-free survival at five years ($p=0.015$ and $p=0.028$). S-phase fraction proved to be a highly significant predictor of prognosis. 35% of patients had low SPF tumors (SPF < 12). The probability of disease-free survival five-years post-recurrence was 100% for the low SPF tumor patients and 32% for patients with high SPF tumors ($p=0.00005$). DNA-ploidy and S-phase fraction were combined to define favorable and unfavorable flow profiles (favorable = DNA-diploid, SPF < 12). Actuarial disease-free survival five-years post-recurrence was 100% for patients with a favorable flow profile and 36% for patients with an unfavorable flow profile ($p=0.0002$). This data suggests DNA flow cytometric measurements at relapse strongly correlate with outcome for patients suffering local recurrence following conservative treatment for early stage breast cancer.

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THE USE OF DNA FLOW CYTOMETRY AS A PROGNOSTIC INDICATOR AFTER LOCAL RECURRENCE IN CONSERVATIVELY TREATED CLINICAL STAGE I AND II BREAST CANCERS.

Introduction

Breast cancer is the most common malignancy among western women with 124,000 new cases reported each year in the United States.[1] Although each American woman has a one in ten risk of developing the disease, early detection of breast lesions with self-examination and screening mammography appear to have increased the number of women for whom cure is possible. Currently, a number of local treatment options, ranging from modified radical mastectomy to breast conserving surgery with radiation therapy, are available. In addition, systemic chemotherapy can be added to delay, if not prevent, distant metastases. Rationale and justification for the use of conservative treatment for early stage breast cancer can be derived from numerous retrospective as well as prospective studies.[2-11]

Patients whose breast cancer has been conservatively treated have a protracted risk for local recurrence. Following local recurrence, salvage mastectomy can be performed offering 59-84% survival at five years.[12-15] Even though many qualitative

clinical and pathological variables have been associated with prognosis at the time of local recurrence, quantitative variables are lacking.[16-20] DNA flow cytometric (DNA-FC) parameters successfully predict prognosis for patients with primary breast cancer.[21-30] The research summarized in this thesis investigates the prognostic value of DNA-FC for women who have experienced local failure following conservative treatment of stage I and II breast cancers.

TREATMENT OPTIONS FOR EARLY BREAST CANCER

Since Roman times, early breast cancer has been considered a local problem amenable to surgical treatment. Until recently, the Halsted radical mastectomy, developed by William Stewart Halsted at the Johns Hopkins Hospital during the 1890's, has remained the standard treatment for early breast cancer in the United States. This procedure assumes breast cancer is initially a local disease, and includes surgical removal of the breast, pectoral fascia, pectoralis major and minor muscles and in most cases complete axillary dissection. The modified radical mastectomy is now the most commonly applied technique for breast amputation. In addition to removal of the cancerous breast, the underlying pectoralis minor muscle, pectoralis fascia and in most cases draining axillary nodes are removed.

With publication in the mid 1960's of the first reports of primary breast irradiation, a new era in breast cancer treatment began.[31-34] These early reports of conservative treatment with limited surgery and primary irradiation were limited to patients who had either refused a radical mastectomy, were elderly or too ill for surgery or had locally advanced disease unsuitable for mastectomy. The reports were

noteworthy as they demonstrated long term survival was possible for at least some patients treated with conservative surgery and primary radiotherapy This led to experimental trials of conservative treatment and subsequently several definitive prospective studies in the United States and Europe which have established the efficacy of conservational surgery with radiation therapy (CS+p°RT) for the treatment of early breast cancer.

EVIDENCE JUSTIFYING THE USE OF CONSERVATIVE TREATMENT FOR EARLY BREAST CANCER

During the last decade, CS+p°RT has been used increasingly for the treatment of early stage breast cancer. This treatment involves excision of the malignant breast lesion, preserving the breast's cosmetic appearance, and departs from the historical perspective of breast cancer controlled by ablative local surgery. Such removal of the lesion is followed by definitive irradiation of the involved breast with or without irradiation of the regional axillary, internal mammary and supraclavicular lymph nodes.

The first studies of conservative treatment for early stage breast cancer were necessarily retrospective. The largest of these are summarized in table 1. Drawing conclusions from these studies is hindered by differences in patient selection, mode of staging, surgical technique, dose of radiation and statistical methods employed. Yet, all demonstrate disease-free and overall survival following CS+p°RT similar to that achieved with mastectomy.[2]

The Yale-New Haven Hospital experience is typical of the retrospective data supporting the use of CS+p°RT as an option for the treatment of early stage breast cancer.[5] This series included 433 women treated with CS+p°RT at the Yale-New Haven Hospital between 1962-1984 of which 70% were clinically stage I and 30% were stage II. All patients were followed for a minimum of five years.

The conservational surgical technique was limited to excision of local tumor with a rim of grossly normal tissue, without a specific requirement for microscopically free surgical margins. Axillary dissections were not routinely performed. Radiation therapy was defined by tangential fields to the breast with an electron boost to the tumor bed to a total median tumor dose of 6400 cGy. The regional nodes were treated in the majority of patients by separate internal mammary and supraclavicular / axillary portals to a total median dose of 4600 cGy. Most patients did not receive adjuvant systemic therapy.

Five-year actuarial survival was 86%. The actuarial breast recurrence rate at five years was 8% of which 82% were salvaged with mastectomy or repeated wedge resection. The five-year actuarial survival following breast only recurrence was 59% and the five-year disease-free survival was 65%.

The results of the Yale experience together with those of the institutions listed in table 1 demonstrated long term survival following CS+p°RT was comparable to that achieved with more traditional radical therapy. The retrospective design of these studies precludes an absolute conclusion but justified randomized prospective trials comparing the two types of therapy.

In order to establish CS+p°RT as an alternative treatment for early breast cancer, five well-designed randomized prospective trials, as summarized in table 2, were undertaken.[3,4,6,35,36] Of these, the National Surgical Adjuvant Breast Project Protocol B-06 (NSABP B-06) is most representative.[6] 1,843 patients with clinical stage I and stage II breast cancer enrolled between April 1976 and January 1984 and were randomly assigned to one of three groups receiving treatment with either mastectomy alone, or conservational surgery with or without primary radiotherapy. All patients underwent axillary dissection. Those with positive nodes received adjuvant chemotherapy. Radiotherapy was delivered to the breast only to a total dose of 5,000-5,300 cGy, without a boost to the tumor site.

Among node negative patients treated with mastectomy, five-year disease-free survival was 71.9% compared to 81.4% for the CS+p°RT group. Overall, survival was 81.7% in the mastectomy group and 90.7% in the CS+p°RT group. The five-year actuarial recurrence rate for patients treated with CS+p°RT was 7% compared to a rate of 32% in patients treated with conservational surgery alone. Life table estimates indicated treatment by conservational surgery, with or without breast irradiation, resulted in disease-free, distant disease-free and overall survival at five years that did not statistically differ from that of patients treated with traditional mastectomy. This study did, however, show a trend towards a longer disease-free and overall survival for patients treated with CS+p°RT.

Several studies have investigated the cosmetic sequelae of conservative treatment. Delouche, at the Centre de Charlebourg in Paris, examined 330 patients

conservatively treated for clinical stage I and II breast cancer and followed for a minimum of two years.[7] In 37.6% of these patients, the treated breast appeared to be essentially identical to the untreated breast. In 39.1%, minor sequelae of treatment were apparent, including breast asymmetry, modest subcutaneous induration or skin changes due to radiotherapy. More marked changes were noted in 16.7%, corresponding to significant deformation of the treated breast and 6.7% of the patients had unacceptable results, representing either a cosmetically unsightly or painful shrunken breast. Similarly, follow-up of five years for 239 conservatively treated patients was obtained by the Joint Center for Radiation Therapy (JCRT) at Harvard University.[37] Overall cosmetic results were found to deteriorate for the first three years following therapy, after which they stabilized. At five years overall cosmetic results were judged as excellent (little or no change) in 77% of patients, good (minimal, but identifiable changes) in 9%, fair (significant results of radiation therapy noted) in 9%, and poor (severe normal tissue sequelae) in 5%. Poor cosmesis was related to extent of surgery, with patients undergoing more extensive excisions having poorer results and total dose of radiation, with a total dose greater than 5,000 cGy being associated with greater degrees of fibrosis and retraction.

Additional complications of conservative treatment are those experienced during primary irradiation of the treated breast. Acute complications include fatigue, irritation and erythema of the skin. These are self-limited, usually resolving within several weeks of treatment. Significant long term complications are unusual. The JCRT reports rib fractures as the most common (5%) followed by cosmetically significant arm edema (4%), radiation pneumonitis (2%) and rarely, paresthesias or brachial plexus disorders.[37]

In 1990, with strong support for the use of conservative treatment for early breast cancer, a panel of experts convened to provide consensus recommendations for conservative surgical treatment techniques and patient selection criterion.[38] The major criterion for patient selection is feasibility of resecting the primary tumor without major cosmetic deformity. Patients not considered appropriate candidates include those with two widely separated primary tumors in the same breast and patients whose mammography reveals diffuse micro-calcifications. Surgical removal of the tumor should be performed to achieve grossly negative margins of resection with verification of microscopically free margins determined on permanent histologic sections after inking of the specimen's borders. Further resection in the case of positive margins is recommended. Axillary dissection, (levels 1-2), provides valuable prognostic information for patients with primary breast cancer and may be routinely employed. It is recommended the entire breast be irradiated to a total dose of 4,500-5,000 cGy, with a boost delivered to the region of the tumor to doses of 1,000-1,500 cGy. It is noted, higher doses of radiation may result in an unacceptable degree of fibrosis and retraction. Future research aimed at refining existing prognostic factors and identifying subgroups most likely to benefit from further adjuvant therapy is encouraged.

LOCAL RECURRENCE IN CONSERVATIVELY TREATED PATIENTS

Having established CS+p°RT as an option for the treatment of early breast cancer, data accumulated from retrospective and prospective studies was analyzed to define the natural history of breast cancer treated with conservative therapy. One initial conclusion derived from such analyses was that local recurrence in conservatively treated patients occurred over a protracted time interval. Unlike patients treated with

mastectomy, most of whom recur within three years, conservatively treated patients are at constant low risk of recurrence for many years following their initial treatment.[39] A collaborative study, conducted at the Princess Margaret Hospital, the Institut Curie and the Marseilles Cancer Institute, demonstrated a constant risk of local recurrence over a fourteen year period.[12] In the Yale series, the breast recurrence rate at five years was 8%, consistent with the 1-2% annual risk of recurrence reported by most institutions.[5] Table 3 summarizes the long risk periods reported for conservatively treated patients.

The continued risk of local recurrence in conservatively treated patients reinforces the need to emphasize rigorous follow-up, as patients treated with breast preserving therapy retain breast tissue at risk for developing a second primary tumor or a recurrence of the original tumor from residual malignant cells which survived the initial treatment.

Since the majority of local recurrences in conservatively treated patients occur at or near the primary site, most are probably recurrences of the primary tumor. 67% of the recurrences in the Yale series occurred at the same site, defined as at or near the original primary site, while 33% were observed elsewhere in the breast.[5] Analysis of recurrences in the JCRT series, after four and a half years of follow-up, revealed 57% occurring directly at the site of the primary, 33% occurring at the edge of the boosted volume and 10% occurring elsewhere in the breast.[40] Similarly, the Marseille group reported 79% of their recurrences occurring in the vicinity of the primary tumor bed but noted that with longer times to recurrence, an increasing percentage were found elsewhere in the breast.[41] Based on such observations, they have suggested that tumors recurring late should be considered second primary tumors.

Pathological examination of recurrences undertaken by the JCRT and the NSABP B-06 project, noted identical or similar histologies among nearly all primaries and local recurrences found at or near the original site.[17,42] Review of primary and recurrence material from the 24 local recurrences treated at Gustave-Roussy revealed in all cases unchanged histological subtype and little or no change in tumor grade.[13]

FACTORS PREDICTING LOCAL RECURRENCE IN CONSERVATIVELY TREATED PATIENTS

Extensive efforts have been made to identify factors which can predict which patients will have a high risk of local recurrence following conservative treatment.[8-11,13,41,43-46] To date, these predictive factors fall into two categories: factors related to tumor burden and factors related to intrinsic tumor biology.

Conservational therapy aims to preserve the cosmetic appearance of the cancerous breast; therefore, it is conceivable that micro-foci of malignant cells remain in the affected breast after tumor excision. In this case, the primary radiotherapy employed after excision should help eliminate the remaining tumor cells. Extent of initial conservational surgery and degree of intra-ductal carcinoma in the primary, as well as the dosage and timing of primary irradiation and other clinical and pathological factors have been reported as prognostic of local recurrence.

Patients in the JCRT series having a less-than-excisional biopsy of their primary suffered a higher percentage of local recurrence than those undergoing complete excisional biopsy (35% vs 7%).[40] When primary tissue from patients suffering local failure in the JCRT series was histologically analyzed, tumors noted to have an

extensive intra-ductal component, (EIC), (considered present when intra-ductal carcinoma occupied 25% or more of the area encompassed by the infiltrating tumor and was present in random sections of grossly unremarkable breast tissue or clearly extended beyond the infiltrating edge of the tumor into surrounding breast tissue) were associated with a substantially increased risk for local failure after ten-years of follow-up. Patients without EIC experienced a 3% failure rate at ten-years compared to a 35% failure rate for those with EIC.[45] However, several groups, including Yale and the Institut Gustave-Roussy, have failed to demonstrate a similar predictive value for EIC, while the Institut Curie found EIC slightly correlated with increased local recurrence but also associated with better survival.[13,37]

Patients experiencing local recurrences in the Institut Gustave-Roussy series were analyzed for thirty variables related to breast relapse.[13] Two of the three factors correlated with local recurrence were duration of radiation treatment and time interval between tumor excision and initiation of radiotherapy. Patients whose treatments were delivered in less than six weeks and those who experienced a delay of more than seven weeks between initial biopsy and radiotherapy had a greater risk of local failure.

Analysis of JCRT data revealed the risk of recurrence at the site of the primary was related to the dose of radiation given to the primary site.[40] Recurrences at the original site were rare in patients receiving doses greater that 6000 cGy. Thus, as dosage of radiation increases and interval to treatment following initial biopsy is shortened, the tumor burden remaining after surgery is presumed to be more effectively eliminated and risk of local recurrence diminished.

The Yale series demonstrated patients with positive axillary nodes had a lower rate of local recurrence.[48] These patients also tended to be younger, have larger primaries and more aggressive tumor histologies. Given these negative prognostic factors, one would have expected these patients to have a higher local recurrence rate. Instead, they fared better than their node-negative counterparts. It is speculated this may be related to the fact that 88% of node-positive patients in the Yale series received adjuvant systemic therapy in contrast to 8% of the node-negative patients. Here again, the use of adjuvant therapy, in this case chemical and/or hormonal, theoretically eliminated the residual tumor burden following conservative therapy, thereby reducing the risk of local recurrence.

Factors related to tumor aggressiveness have also been associated with the risk of local failure. The NSBABP B-06 analyses showed high nuclear grade associated with increased risk for local recurrence.[49] In the JCRT series, grade 3 tumors were associated with higher percentages of local failure.[50] The Marseille Risk Factor study showed mononuclear cell reaction, a marker of aggressive tumor growth, and high histologic grade were predictors of local failure.[11] The need for more accessible and easily reproducible measures of biological aggressiveness, such as DNA-FC analyses or measurements of growth fractions with monoclonal antibody techniques, is proposed. To date, only microscopic examination of tumor specimens can be used to assess risk of local failure secondary to tumor aggressiveness in conservatively treated patients.[11]

PROGNOSIS FOLLOWING LOCAL RECURRENCE

Prognosis following local recurrence for patients treated with conservative therapy differs from that of patients treated with mastectomy. 33-50% of patients experiencing chest wall recurrence after mastectomy have concurrent distant metastases. In contrast 9-25% of patients experiencing local failure following conservative treatment have concurrent distant disease.[16] Patients who recurred in the chest wall after mastectomy and subsequently received radiation therapy where followed by the Joint Center at Harvard. Five-year disease-free survival in this group was 30% and decreased to 7% at ten years.[16] This trend is confirmed by several studies which report survival following chest wall recurrence in patients treated with primary mastectomy ranges from 21-36% at five years and 5-26% at ten years.[51-54] In contrast, various studies report five-year disease-free survival following local recurrence in conservatively treated patients between 59-84% and ten-year disease-free survival ranging from 50-65%.[12-15] Researchers at the Institut Gustave-Roussy and Princess Margaret Hospital failed to demonstrate any difference in overall survival between conservatively treated patients who experienced local failure and those who had never had a local recurrence.[13,55] Table 4 provides survival data following local recurrence from several representative series.

The optimal treatment for patients who experience local recurrence following conservative therapy is not yet known. Currently, most patients undergo salvage mastectomy, although in some cases further conservative treatment is employed.[16] To date, the role of adjuvant chemotherapy and hormonal therapy in this population remains undefined.

To aid in the management of patients experiencing local recurrence after conservative therapy, efforts have been made to find reliable predictors of prognosis following failure. Currently available prognostic indicators are limited to clinical observations. Those most consistently reported are time to recurrence, extent and site of recurrence and histology of recurrence.

In the Yale series, patients recurring four or more years after primary treatment had a significantly better prognosis than patients recurring sooner.[20] Similarly, patients treated in the Marseille series were found to have a favorable prognosis (84% survival at five years) if they recurred five or more years after primary treatment.[41] At the Institut Curie, recurrences occurring within three years after primary therapy were more often associated with concurrent nodal involvement and distant metastases and, as a result, poorer prognosis.[20] The University of Pennsylvania series demonstrated a statistically insignificant trend towards poorer prognosis among early recurrences.[15] The JCRT failed to show any difference in survival among patients who recurred after two or more years compared to those recurring in less than two years.[16]

Various measures assessing qualitative features of locally recurrent tumors have been predictive.[15,16,20,41] Site and extent of local recurrence was predictive in the Yale series.[20] Localized tumors, recurring elsewhere in the breast and measuring less than 3 cm, were associated with a better prognosis. Similar findings have been reported by the University of Pennsylvania, Marseille and Gustave-Roussy.[13,15,41] Others have failed to demonstrate the prognostic value of these indicators.[16]

Examination of histologic features of recurrent tumors has not been uniformly helpful in predicting outcome. In the JCRT series, patients with purely or predominantly non-invasive recurrent tumors suffered no further recurrences, whereas, 38% of patients with predominantly infiltrating tumors, experienced some further recurrence.[16] Histology of the recurrent tumor, assessed somewhat differently, was not prognostic in the Yale series.[20]

PROGNOSTIC INDICATORS USED FOR PRIMARY BREAST CANCER

At best, currently available data provides a number of clinical observations which can be associated with prognosis following local recurrence in the conservatively treated patient. Histologic features of the recurrent tumor are the only objective measures available, but data examining this potential predictor is scarce, inconclusive and hindered by potential problems of reproducibility. In contrast, prognosis for patients with primary breast cancer has been successfully predicted using a number of quantitative as well as qualitative factors.

The Surveillance, Epidemiology and End Results (SEER) program of the National Cancer Institute has followed 24,740 breast cancer patients through tumor registries in five states.[56] The results of this study indicate size of primary and lymph node status are independent but additive prognostic indicators for primary breast cancer. As tumor size increases, survival decreases regardless of nodal status. Likewise, as nodal involvement increases, survival status decreases regardless of tumor size. Patients with primaries less than 2 cm in diameter and negative lymph nodes have the most favorable prognosis with 96.3% five-year survival whereas patients with

primaries larger than 5 cm and positive axillary nodes have a survival at five years of only 45.5%.

In addition to clinical measures of prognosis, several quantitative measures have proven to be valuable indicators of prognosis in primary breast cancer. Steroid hormone receptor status has well established independent predictive value.[57] In patients with negative lymph nodes, positive estrogen receptor status is associated with a better overall and disease-free survival. For patients with positive nodal involvement progesterone receptor status is a strong prognosticator.[58] In this case, lymph node-positive patients with positive progesterone receptor status have a better disease-free survival than their progesterone receptor negative counterparts.

Currently evolving quantitative prognostic indicators for primary breast cancer include measures of DNA content and cellular proliferation, obtained using techniques of DNA-FC.[27] Overall, tumors with DNA values within the limits of normal tissue correlate with favorable prognosis while tumors with high proliferative indices are associated with poor outcome.[21-26,28-30]

TECHNIQUES OF CELL ANALYSIS USING DNA FLOW CYTOMETRY

While tumor cell proliferation can be measured by both the histologically derived mitotic index and the autoradiographically derived thymidine labeling index, the technique of DNA-FC provides rapid measurement of cell proliferation without the constraints of fresh tissue or labor intensive histologic analyses. In addition, measures of DNA content are concurrently obtained with DNA-FC analysis. Thus, DNA-FC

analysis of fresh or archival tumor cells allows direct measurement of DNA content (DNA-ploidy) and indirect measurement of tumor cell proliferation (S-phase fraction, SPF). When the measured DNA content of tumor cells is equal to that found in normal cells the tumor is defined as DNA-diploid. If the DNA content is either greater or less than normal, the tumor is defined as DNA-aneuploid.

Hedley developed the technique for analyzing paraffin-embedded archival tissue with DNA-FC after observing the impressive degree of cellular preservation in paraffin-embedded tissue slices which were dewaxed and rehydrated.[59] Once archival tumors cells are rehydrated, they are processed in a manner similar to that previously established for cytometric analysis of fresh tissue. Intact tumor cells are treated with a digestive enzyme, such as trypsin or pepsin, with subsequent tumor lysis. The freed cellular nuclei are then tagged with an appropriate DNA fluorochrome and run through the DNA flow cytometer.

The flow cytometer has four components: a light source, usually a laser, a sample chamber and optical assembly, a set of associated electronics that convert light impulses to digital signals and a computer system that controls instrument operations, collects data and performs analytical routines.[60] Once a cell sample is prepared, it is added to the sample chamber and at a rate of approximately 10,000 cells per second, passes single file past the light source. As cells pass the laser, light is scattered in all directions. Light which is deflected forward is referred to as forward angle light scatter (FALS) and is directly proportional to the size of the passing particle, assuming the particle is a homogeneous sphere. Light which is orthogonally deflected is referred to as 90 degree light scatter (90°LS). It is reflected from internal cellular structures and is

indicative of cell granularity. Fluorescent emissions from DNA specific probes excite the system's lasers and generate histograms from which the DNA content of resting and dividing cell populations is determined. The DNA flow cytometric histogram from a normal cell population will contain a single G0/G1 peak representing most of the sample's cells in the resting phase of the cell cycle. Smaller peaks will be generated by cells in the proliferative G2/M and S-phases.

A cell population is defined as DNA-aneuploid if it has two or more G0/G1 peaks with the second being at least 10% of the first.[61] DNA index is the calculated ratio of the abnormal G0/G1 peak to the normal G0/G1 peak. Cell cycle analysis computer programs divide DNA histograms into their constituent parts of G0/G1, G2/M and SPF by assessing the relative distribution of DNA. Representative DNA-diploid and DNA-aneuploid histograms appear in figures 1 and 2 respectively.

THE USE OF DNA FLOW CYTOMETRY AS A PROGNOSTIC INDICATOR FOLLOWING LOCAL RECURRENCE

Measures of DNA content and cellular proliferation are valuable prognostic indicators for a number of solid and non-solid tumors.[62] Methods have been successfully developed to apply DNA-FC analysis on paraffin-embedded archival tissue. The reproducibility and correlation between data derived from fresh and archival tissue has been verified by Hedley and others.[59,63-68] Applying this technology to the readily available archival tissue obtained from patients who experienced local recurrence in the Yale series should provide an indication of the prognostic value this measure holds for this population.

Methods and Materials

PATIENTS

433 patients with primary clinical stage I and stage II breast cancer were treated with conservational surgery and primary irradiation at the Yale-New Haven Hospital between January 1962 and December 1984. Clinical staging was in accordance with the guidelines of the American Joint Committee/ Union Internationale Contre Le Cancer. Conservational surgery involved excisional biopsy of the breast lesion with no specific attempt made to obtain microscopically free surgical margins, although the surgical specimen generally contained a rim of normal breast tissue around the tumor. Axillary dissection was not routinely performed. Most patients did not receive adjuvant systemic therapy although multi-agent chemotherapy or hormonal therapy was used in some patients with pathologically positive nodes.

Radiation therapy was delivered using 4-6 MeV linear accelerators with tangential fields directed at the breast with an electron boost to the tumor bed to a total median dose of 6400 cGy. Radiation was delivered in 200 centigrade per day fractions. Wedges were routinely employed to optimize dose homogeneity throughout the breast tissue.

The regional nodes were treated in the majority of patients. In patients not undergoing axillary dissection, the internal mammary, supraclavicular and axillary nodes were irradiated. Internal mammary nodes were irradiated with alternating photons and 13 MeV electrons with a separate field to a total median dose of 4600 cGy

specified at a depth of 3 cm. Occasionally the internal mammary nodes were included in the tangential fields. Supraclavicular and axillary nodes were treated with a separate half-blocked anterior field angled 10-15° off of the spinal cord. The field extended medially from the midline angled off the spinal cord and cleared the entire axillary contents laterally.

In patients undergoing axillary dissection, the internal mammary chains and breast were treated as described above. The supraclavicular field, however extended only from the midline to approximately the medial border of the humeral head laterally, thus sparing the lower axillary contents in both node positive and node negative patients. Radiation treatment was delivered over a six to seven week period. Figure 3 schematically represents these treatment policies.

Patients were seen in follow-up by both the radiation oncologist and referring surgeon at three to six month intervals. Routine mammograms and chest X-rays were performed annually. Follow-up liver scans and bone scans were not performed unless clinically indicated. Disease and survival status for each patient is routinely updated with documented follow-up visits, correspondence with the patient and her surgeon or internist and through data available from state tumor registries.

Charts were retrospectively reviewed in an effort to document all patients experiencing local recurrence. 50 patients experienced recurrent tumor in the conservatively treated breast prior to June 1990. An effort was made to obtain tissue samples from the recurrent breast tumors of these patients. Tissue could be retrieved for 76% of the recurrent tumors.

DNA FLOW CYTOMETRY

DNA-FC analysis was carried out on all available tissue samples. Cell preparation was carried out as follows. 50 μm sections were obtained from each paraffin-embedded sample. An additional 6 μm cut was made from each section and a slide prepared to verify the presence of tumor cells in each section. Sections were deparaffinized with Americlear solution, followed by stepwise hydration in ethanol (100%, 95%, 70%,50%) and distilled water. Cells were then treated with pepsin and lysed. Nuclear material was tagged with propidium iodide and analyzed with an Epics Profile flow cytometer (Coulter Electronics Inc.) using 488 nm excitation. Both forward and 90 degree light scatter gates were used to exclude debris signals. DNA index and cell cycle distribution were calculated using the Modfit software program. The mean coefficient of variation for the entire sample was 5.6 (median 5.2).

STATISTICAL ANALYSIS

Statistical analyses were performed employing the PRODAS statistical package from Conceptual Software Inc. on a Zenith 386 IBM compatible computer. The significance of survival differences between patient groups was calculated according to the Cox Life Table and Survival Analysis Model. The Cox Life Table Regression Model was employed for univariate analyses.

Results

PATIENT POPULATION

As of January 1990, 433 patients, with a minimum follow-up of five years and a median follow-up of 8.21 years, have received conservative treatment at the Yale-New Haven Hospital. Characteristics of the treated population appear in table 5. The median age of treated patients is 55.0 years (range, 20-89 years). 70% were treated for clinical stage I breast cancer. 43% underwent axillary dissection. 9% received adjuvant hormonal therapy and 12% received adjuvant chemotherapy. The overall five-year actuarial survival rate for the population is 0.86 ± 0.02, the actuarial five-year disease-free survival rate is 0.82 ± 0.02.

PATIENTS WITH LOCAL RECURRENCE

Patient status, as of January 1990, appears in table 6. 82% of patients in the entire population remain alive. 50 patients have experienced local failure with a five-year actuarial breast recurrence rate of 0.08 ± .02. 44 of the recurrences were confined to the breast, 6 patients had coincident distant disease. Follow-up is available for a median of 5.06 years following recurrence.

Flow data was obtained from 100% of available tissue samples. Recurrent tumor tissue was available for 38 patients (76%). Patients for whom tumor tissue is available do not differ significantly from the total recurrent population in terms of age, original clinical stage, original nodal status, mode of recurrence detection, interval to

recurrence, site of recurrence, extent of recurrence, adjuvant therapy, salvage therapy and disease status (table 7).

Figures 4 and 5 depict actuarial curves of overall and disease-free survival for all patients experiencing local recurrence and for patients with available flow data. Median follow-up post-recurrence for patients with flow data is 5.8 years. For all recurrences the five-year actuarial survival post-recurrence is 59% and five-year actuarial disease-free survival post-recurrence is 65%. For patients with available flow data, overall actuarial survival five-years following recurrence is 48%. Actuarial disease-free survival for patients with available flow data is 58% at five-years following recurrence.

Univariate analysis shows extent of recurrence and time to recurrence are predictive for five-year disease-free survival among the patients with available recurrent flow data ($p = 0.000$ and 0.007 respectively). Extent of recurrence predicts overall survival in this group ($p = 0.005$), while time to recurrence approaches statistical significance ($p = 0.06$).

DNA-PLOIDY

Most recurrent tumors (62.8%) were DNA-diploid. Univariate analysis, using the Cox Life Table Regression Model, shows DNA-ploidy is predictive of overall survival and disease-free survival five-years after local failure ($p=0.015$ and $p=0.028$, respectively). Overall actuarial survival, calculated according to the Cox Life Table and Survival Model, at five-years post recurrence is 63.0% in patients with DNA-diploid

tumors and 14.9% in patients with DNA-aneuploid tumors. Actuarial curves depicting overall survival appear in figure 6. DNA-ploidy, calculated by Chi-square analysis, is positively correlated with distant disease progression ($p < 0.05$). 46% of patients with DNA-aneuploid tumors remain distant disease-free, whereas, 76% of patient with DNA-diploid tumors are free of distant disease progression (table 8).

S-PHASE FRACTION

The median SPF for the sample was 13.8. An SPF value of less than to 12 was used to define tumors with a low proliferative index. 35% of patients had a tumors with a low proliferative index. When subjected to univariate analysis, favorable proliferative index was found to be predictive of overall survival at five-years post recurrence ($p=0.01$) as well as disease-free survival at five-years post recurrence ($p = 0.0002$). Overall, actuarial survival five-years post recurrence is 82.6% in patients with low SPF tumors and 24.0% in patients with high SPF tumors. Actuarial disease-free survival five-years post recurrence is 100% in patients with low SPF tumors and 32.1% in patients with high SPF tumors. Actuarial curves depicting this data are presented in figures 7 and 8. SPF is positively correlated with distant disease progression. ($p <0.01$). 56% of patients with high SPF tumors have had distant disease progression while, 100% of the patients with low SPF tumors remain distant disease free (table 8).

COMBINED DNA-PLOIDY AND S-PHASE FRACTION

The results of the separate analyses of patients according to DNA-ploidy and S-phase were combined to create classifications of tumors with favorable and unfavorable flow profiles. Tumors with a favorable flow profile are defined as DNA-diploid with a SPF less than 12. Those with an unfavorable flow profile are defined as DNA-aneuploid or DNA-diploid with an SPF greater than 12. 13 patients (30%) had favorable flow profiles. Univariate analysis shows favorable flow profile is predictive of overall survival five-years post recurrence ($p=0.03$) and disease-free survival five-years post recurrence ($p=0.00005$). Overall actuarial survival five-years after recurrence is 60% for patients with a favorable flow profile and 24% for patients with an unfavorable flow profile (figure 9). Actuarial disease-free survival five-years after recurrence is 100% for patients with a favorable flow profile and 36% for patients with an unfavorable flow profile (figure 10). Distant disease progression is positively correlated with flow profile ($p < 0.01$). 52% of patients with unfavorable flow profiles had distant disease progression, whereas, 100% of patients with favorable flow profiles remain free of distant disease (table 8).

The distribution of previously established post-recurrence prognostic indicators among patients with favorable and unfavorable flow profiles appears in table 9. The clinical predictors of prognosis, interval to recurrence, site of recurrence and extent of recurrence are all positively correlated with flow profile.

Discussion

Local recurrence in the conservatively treated patient is associated with a prognosis more favorable than that of a patient who has suffered local failure following primary treatment with mastectomy. Following salvage mastectomy, approximately 50% of these patients will remain disease-free and 50% will succumb to progressive disease. Measures of recurrent tumor DNA content and proliferative index obtained through DNA-FC appear to be predictive indicators in this population of patients.

When compared to patients with DNA-diploid recurrences, patients with DNA-aneuploid tumors have shorter overall and disease-free survival. This parameter has not been systematically measured in patients experiencing local failure following conservative treatment. Its prognostic value for primary breast cancer is widely reported.[23,26,28,30,65] Beerman performed DNA-FC on fresh and archival tissue from 690 patients with stage I-III breast cancer followed for a median of seven years and observed that DNA-aneuploidy was associated with shortened overall and disease-free survival.[30] Coulson looked at 74 patients with a median follow-up of three years and found 92% of deaths occurring within three years were in patients with DNA-aneuploid tumors.[26] Others have failed to demonstrate the prognostic value of DNA-FC when measuring DNA-ploidy alone.[29,69] Toikkanen's retrospective examination of tissue from 115 stage I-II breast cancer patients with a median follow-up of twenty-seven years failed to demonstrate any influence on survival with respect to DNA-ploidy.[29] Similarly, Rofagha 's examination of archival tissue from 165 patients with node-negative breast adenocarcinoma, followed for a median of eight years, did not show prognostic significance for measures of DNA ploidy. [69]

Measures of S-phase fraction alone were highly significant predictors of overall and disease-free survival. Using the institutionally accepted cut-off of 12%, patients with low SPF had longer overall and disease-free survival. S-phase fraction has not been previously examined as a prognostic indicator following local recurrence in conservatively treated breast cancer but its utility as a prognostic indicator in primary breast cancer is well documented [21,23,65] Using 9% as a cut off for low SPF, Hatschek measured SPF in the primary tumors of 15 patients who experienced local failure. The patients' initial staging and mode of treatment were not reported. High SPF was associated with an increased, but not statistically significant, risk of recurrence but did significantly correlate with length of survival following dissemination of disease.[24]

DNA-ploidy and S-phase fraction both independently predict outcome following local recurrence. Information derived from these two parameters was combined to assign patients to favorable and unfavorable groups. Patients with "favorable" flow profiles (DNA-diploid, SPF < 12) had significantly longer overall and disease-free survival. This is in agreement with studies in patients with primary breast cancer, which have assigned patients to prognostic groups by combining measures of DNA-ploidy and SPF.[22,23] Clark's examination of breast tissue from 345 node-negative patients with a median follow-up of 4.9 years showed that overall and disease-free survival was longest in patients with DNA-diploid, low SPF tumors.[22] Kallioniemi applied a prognostic index which includes DNA index and S-phase fraction to 294 stage I-III breast cancer patients and found that it correlated with overall survival.[23]

In order to apply data derived from DNA-FC to clinical practice, the reliability of these measures must be considered. The established "gold-standard" for measurement of cell proliferative indices is tritiated thymidine labeling (TLI). This autoradiographic technique cannot be practically applied on a large scale because it is time consuming, expensive and requires fresh tissue. Nonetheless, its value as a powerful independent prognostic indicator in primary breast cancer is strongly supported in the literature.[27] DNA-FC provides information on both DNA content and proliferative index and offers the advantage of rapid tissue analysis which can be performed on fresh or archival tissue. Costa and McDivitt report a positive correlation between S-phase values obtained by DNA flow cytometry and those obtained by tritiated thymidine labeling.[67,70] Kallioniemi's comparison of fresh and paraffin-embedded tumors showed S-phase fractions from both tissue types were positively correlated.[64]

Technical difficulties associated with flow cytometric analysis of paraffin-embedded tissue raise concern about the accuracy of its determinations. Analysis of paraffin-embedded tumors is usually associated with a larger coefficient of variation representing larger proportions of cellular debris in the tissue sample. This raises the concern that some near DNA-diploid tumors are misclassified as DNA-diploid. Hedley looked at 233 frozen tissue samples and compared them to 280 paraffin-embedded breast tumors with similar pathological staging and found the paraffin samples had a higher proportion of tissues with a DNA index of 1.0 (DNA-diploid). Yet, his comparison of 24 frozen and paraffin-embedded samples from the same tumor yielded identical proportions of near DNA-diploid tumors.[65]

The reliability of SPF measures derived from paraffin-embedded tissue has also been called into question.[64] In the case of DNA-aneuploid tumors, overlapping cell-cycle distributions create technical difficulties in determining S-phase fraction which, in contrast, is usually readily evaluable in DNA-diploid tumors. However, in DNA-diploid tumors interpretation of SPF is difficult because the cell-cycle distribution represents both tumor and non-tumor cells. Kallioniemi demonstrated greater intratumor variation in SPF measures derived from DNA-diploid tumors and concludes this results from cytometrically indistinguishable populations of tumor and non-tumor cells in a DNA-diploid tissue sample.[64] He notes, however, that the extent of this problem can be reduced by carefully selecting for analysis only those tissue sections which contain large numbers of tumor cells. Despite these potential technical limitations, DNA-ploidy and S-phase fraction determinations have been consistently correlated with outcome in primary breast cancer.

The implications of the data presented in this study rest in its potential role as a guide for the clinical management of patients experiencing local recurrence following conservative treatment for early stage breast cancer. Unlike primary breast cancer, for which the well defined prognostic indicators of stage, lymph node and receptor status are used to guide treatment, few prognostic indicators are available to help guide the management of patients experiencing local failure following conservative therapy. Axillary exploration at the time of recurrence is of questionable value. Most patients undergoing conservative treatment undergo axillary dissection and irradiation at the time of primary treatment. This makes re-exploration at the time of recurrence a technically difficult, low yield procedure. Although retrieval of a positive lymph node may influence decisions regarding adjuvant systemic therapy, the conclusions derived from

an absence of involved nodes must remain equivocal, due to the necessarily small sample likely to be retrieved from a previously dissected axilla.

The prognostic significance of hormone receptor status in this patient population remains undetermined. Preliminary analysis of receptor status in the Yale series suggests patients with recurrent tumors which are estrogen receptor-negative have poorer survival than patients with estrogen receptor-positive tumors.[20] Unfortunately this data is available for only 33% of the patients experiencing local recurrence. Determination of receptor status by immunohistochemical staining of paraffin-embedded blocks, obtained from the majority of patients experiencing local failure in our series, is currently underway.

Clinical and pathological variables, including time to recurrence, extent and location of recurrence and tumor histology, have been shown to be prognostically significant. Adapting these variables for use as guides for the clinical management of patients following local failure is hampered by their heavy reliance on observer judgment and resulting problems of reproducibility due to inter-observer variability. In contrast, DNA-FC analysis provides readily reproducible quantitative measures which, in our series, were positively correlated with these previously established clinical prognostic factors.

The low overall incidence of local recurrence following conservative treatment for early stage breast cancer results in relatively small numbers of patients available for study. The accumulation of larger samples with longer follow-up will be necessary to firmly establish the prognostic significance of DNA-FC analysis for this population.

When this is achieved, these prognostic factors can be used to design the randomized prospective clinical trials which will be necessary to determine optimal salvage treatment and appropriate adjuvant systemic therapy for patients experiencing local recurrence following conservative treatment.

In summary, DNA flow cytometric measurements of DNA-ploidy and S-phase fraction in local recurrent tumor tissue may help predict overall and disease-free survival following local failure in conservatively treated stage I-II breast cancer patients. In our sample patients with DNA-diploid, low S-phase tumors had an excellent prognosis. With further verification of these findings by others with similar patient populations, the application of this data to the clinical management of patients experiencing local failure can be anticipated.

Table 1
Long Term Survival Following Conservative Treatment of Early Stage Breast Cancer - Retrospective Studies

Series	Dates	Subjects	Follow-up	Overall survival			Disease-free survival		
				5 years	10 years	15 years	5 years	10 years	15 years
Yale [26]	1962-1984	433 stage I-II breast cancers	median 8.21 years	86%	--	--	92%	--	--
MDAH [10]	1955-1985	536 stage 0-IIB breast cancers	median 5.34 years	87%	73%	--	90%	82%	--
Charlesbourg [7]	1956-1979	410 stage I-II breast cancers	median 11.0 years	82%	62.5%	45.4%	--	--	--
Henri Mondor [8]	1961-1979	493 stage I-II breast cancers	mean 10.0 years	--	68%	--	--	67%	--

Table 2
Mastectomy vs. Conservative Treatment for Early Stage Breast Cancer -- Randomized Prospective Trials

Series	Dates	Subjects	Follow-up	Overall Survival		Disease-free Survival	
				Mastectomy	Conservative Treatment	Mastectomy	Conservative Surgery
Milan [3]	1973-1980	349 Halsted mastectomy 350 Quadrantectomy with radiation therapy	8 years	83%	85%	77%	80%
NSABP B-06 [6]	1976-1978	586 Total mastectomy 625 Segmental mastectomy with radiation therapy	5 years	81% node-neg. 66.4% node-pos.	91.6% node-neg. 75.2% node-pos.	71.9% node-neg. 57.9% node-pos.	81.4% node-neg. 57.5% node-pos.
Gustave-Roussy [4]	1972-1980	88 Mastectomy 91 Tumorectomy with radiation therapy	5 years	91%	95%	74%	85%
Guy Hospital* [35]	1981-1986	185 Modified radical mastectomy 214 conservative surgery and radiaton therapy	NR	NR	NR	NR	NR
NCI* [87]	1979-1985	95 Mastectomy 102 Conservative surgery and radiation therapy	2.6 years	NR	NR	NR	NR

* no difference reported in overall and disease-free survival between mastectomy and conservative treatment groups

Table 3
Local Recurrence Rates in Conservatively Treated Early Stage Breast Cancer

Series	Subjects	Follow-up	Breast Recurrence Rate
GUSTAVE-ROUSSY[13]	436	5.0 years	7% at five years
MDAH [10]	536	5.3 years	9% at five years
JCRT [40]	607	6.3 years	9% at five years
YALE [20]	433	8.2 years	8% at five years
MARSEILLE [9]	1593	11 years	7% at five years

Table 4
Survival Following Local Recurrence in Conservatively Treated Patients

Series	Number of Recurrences	Follow-up Following Recurrence	Overall Survival Following Recurrence
Yale [20]	50	5.0 years	59% at five years
JCRT [16]	90	2.8 years	69% at eight years
Univ. of Pennsylvania* [15]	65	1.6 years	84% at five years
Marseille [14]	178	not reported	70% at five years

*Survival calculated for patients undergoing salvage mastectomy

Table 5
Patient Population Characteristics
N=433

Characteristic	Frequency	Percentage
AGE		
< 50 years	159	37
> 50 years	274	63
CLINICAL STAGE		
Stage I	301	70
Stage II	132	30
PATHOLOGICAL NODAL STATUS		
Node-negative	128	30
Node-positive	57	13
No axilllary dissection	248	57
ADJUVANT THERAPY		
Chemotherapy		
No	379	87
Yes	54	12
Hormonal Therapy		
No	393	91
Yes	40	9

*Mean age is 54.0 years (Median 55.0 years).
Mean follow-up is 9.1 years (Median 8.21 years)

Table 6
Patient Status as of January 1990
N=433

Status	Frequency	Percentage
ALIVE	356	82
DEAD	77	18
FAILURE		
Treated breast alone	44	10
Treated breast with distant metastases	6	13
Lymph nodes	12	11
Distant metastases	47	3

Table 7
Comparison of Characteristics of All Patients Experiencing Local Recurrence and Those with Flow Data Available

Characteristic	All Recurrences N=50		Recurrences with Flow Data N=38		
	frequency	%	frequency	%	p
AGE AT DIAGNOSIS					
< 50 years	25	50	14	37	NS
>50 years	25	50	24	63	
CLINICAL STAGE					
Stage I	37	74	28	74	NS
Stage II	13	26	10	26	
ORIGINAL PATHOLOGICAL NODE STATUS					
Node-negative	13	26	8	21	NS
Node-positive	9	18	7	18	
Unknown	28	56	23	61	
MODE OF DETECTION					
Physical exam only	19	38	16	42	NS
Mammography only	14	28	10	26	
Physical exam and mammography	14	28	10	26	
Physical exam, mammography negative	3	6	2	5	
SALVAGE THERAPY					
Modified radical mastectomy	21	42	15	39	NS
Simple mastectomy	18	36	14	37	
Lumpectomy	7	14	4	10	
Biopsy	4	8	4	10	
INTERVAL TO RECURRENCE					
< 4 years	24	48	19	50	NS
> 4 years	26	52	19	50	
SITE OF RECURRENCE					
Same site	36	72	28	74	NS
Elsewhere	14	28	10	26	
EXTENT OF RECURRENCE					
Localized	38	76	28	74	NS
Diffuse	12	24	10	26	
ADJUVANT THERAPY					
Chemotherapy	15	30	11	29	NS
Hormonal therapy	8	16	8	21	
None	27	54	19	50	
DISEASE STATUS					
Alive, No Evidence of Disease	27	54	17	44	NS
Alive, with Disease	5	10	5	13	
Dead, No Evidence of Disease	2	4	2	5	
Dead, with Disease	16	32	14	37	

Table 8
Frequency of Distant Disease Progression by Flow Parameters at Recurrence
N=38

Flow Parameters	No Distant Disease		Distant Disease Progression		
	frequency	%	frequency	%	p
DNA-PLOIDY					
DNA-Diploid	19	76	6	24	$p < 0.05$
DNA-Aneuploid	6	46	7	54	
S-PHASE FRACTION					
SPF < 12	15	100	0	0	$p < 0.01$
SPF > 12	10	43	13	57	
FLOW PROFILE					
Favorable (D, <12)	13	100	0	0	$p < 0.01$
Unfavorable	12	48	13	52	

Table 9
Distribution of Post-recurrence Prognostic Indicators Among Patients with Favorable and Unfavorable Flow Data*
N=38

Characteristic	Favorable Flow Profile N=14		Unfavorable Flow Profile N=24		
	frequency	%	frequency	%	p
INTERVAL TO RECURRENCE					
< 4 years	3	21	16	67	$p < 0.01$
> 4 years	11	79	8	33	
SITE OF RECURRENCE					
Same site	7	50	21	87	$p < 0.05$
Elsewhere	7	50	3	13	
EXTENT OF RECURRENCE					
Localized	14	100	14	58	$p < 0.01$
Diffuse	0	0	10	42	

* Favorable Flow Profile = Diploid with SPF $\leq$ 12
Unfavorable Flow Profile = Aneuploid/ Diploid with SPF >.12

Figure 1
DNA-diploid Histogram

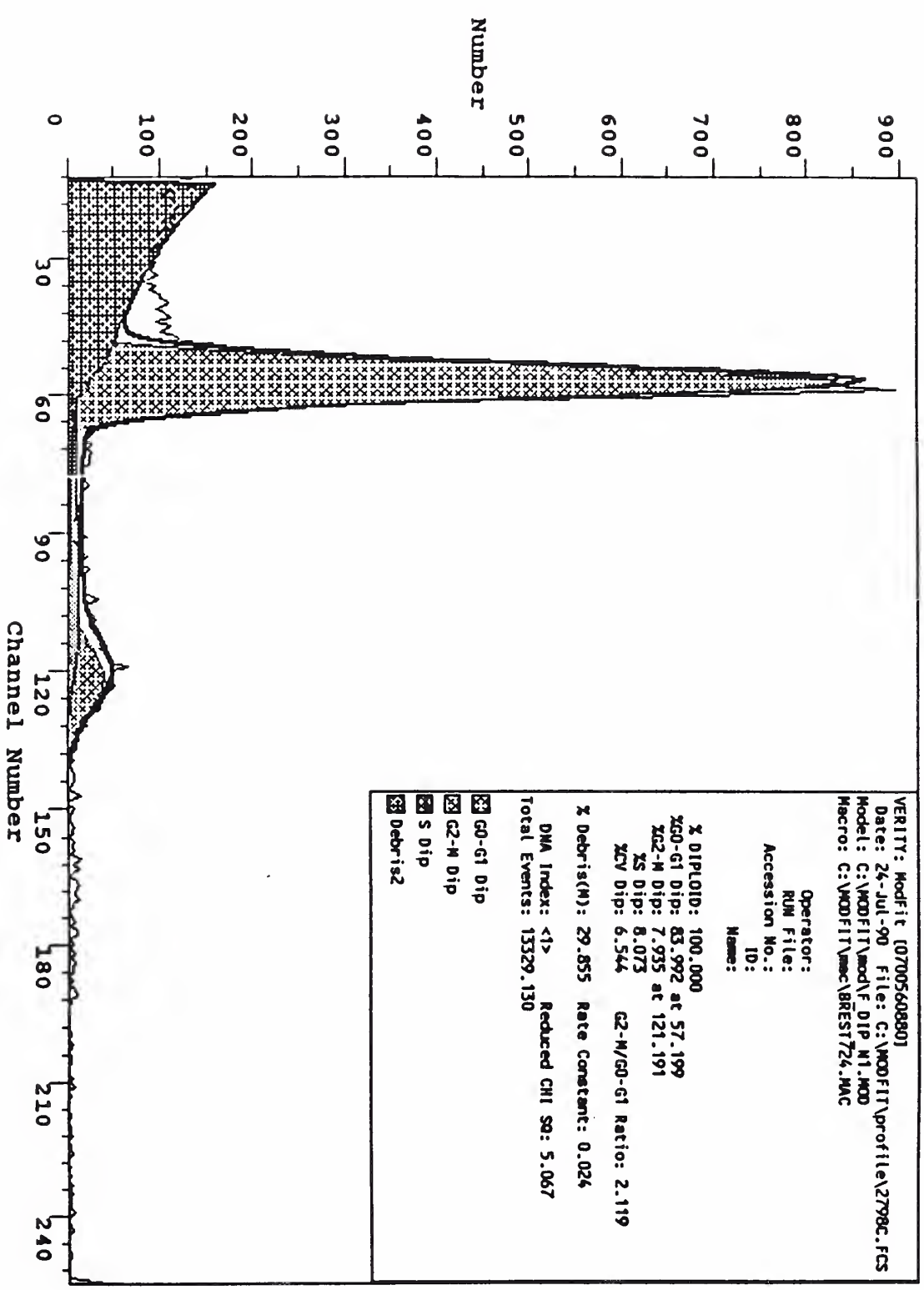


Figure 2
DNA-aneuploid Histogram

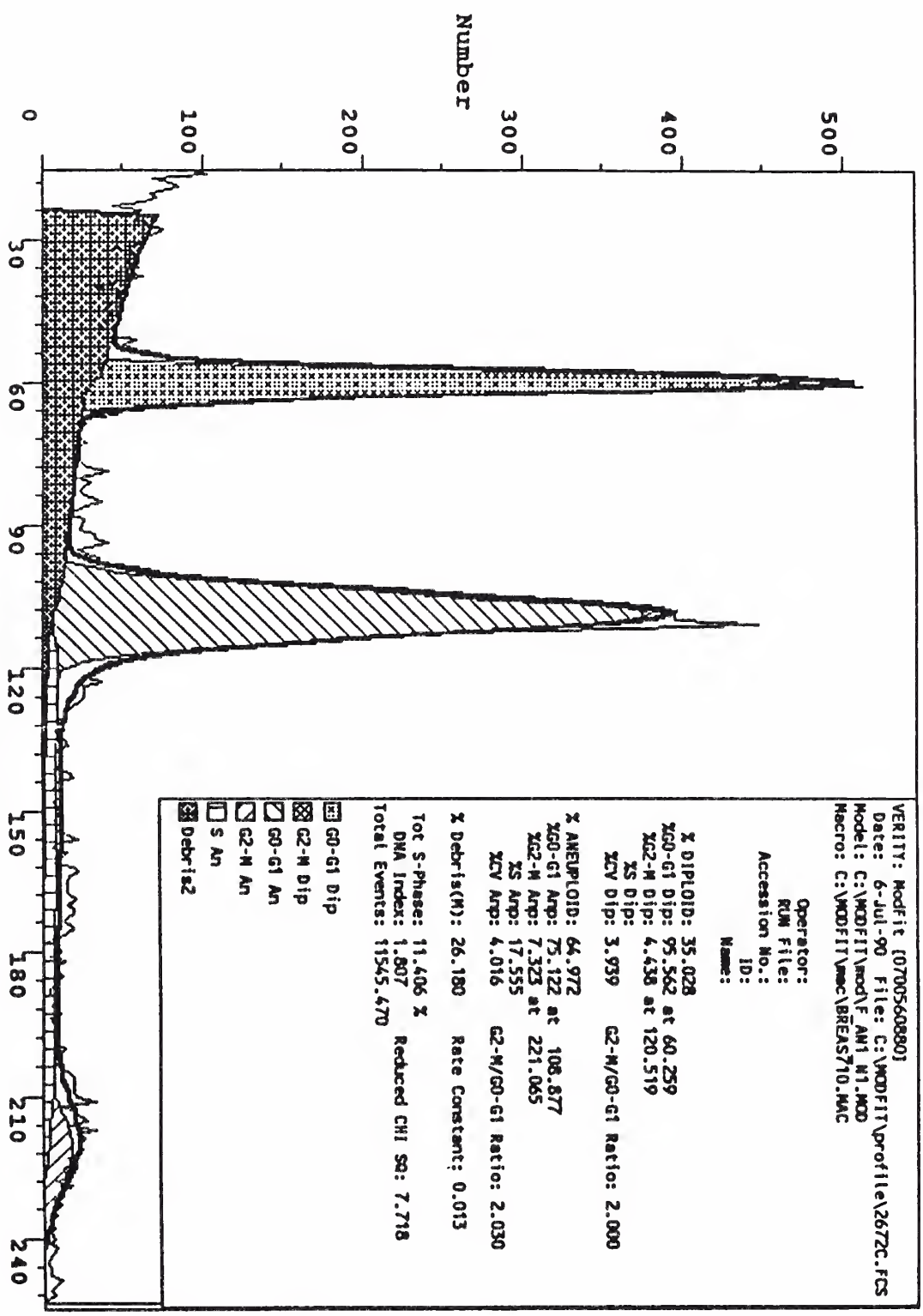


Figure 3
Schematic Representation of Radiation Treatment Policy

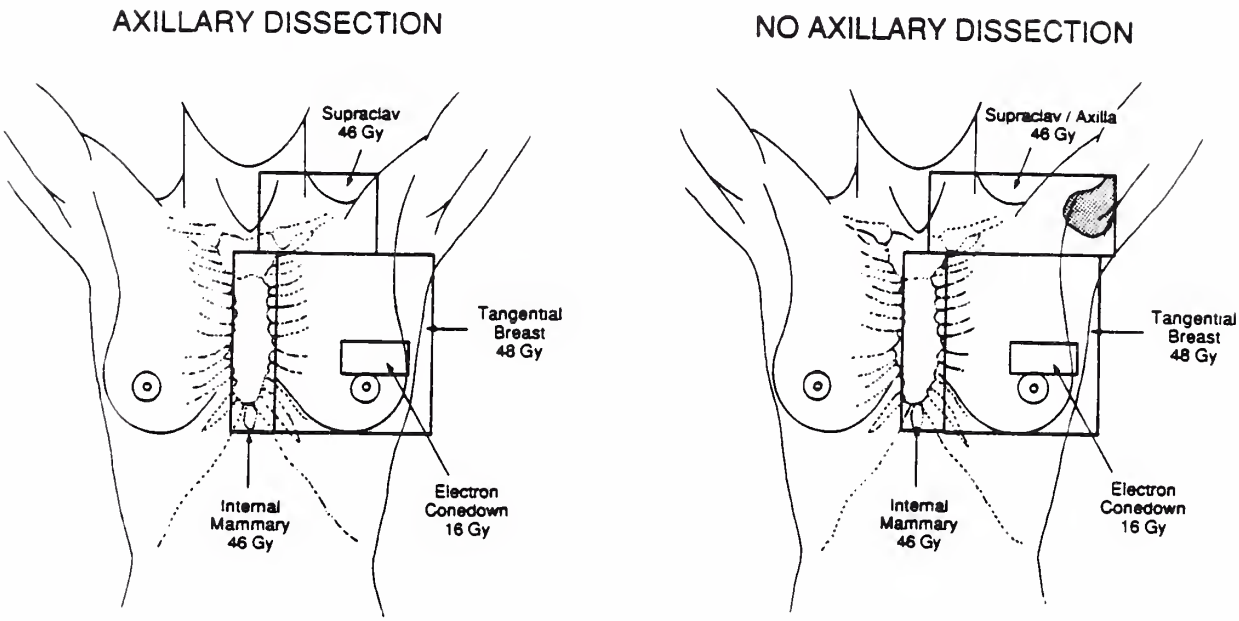
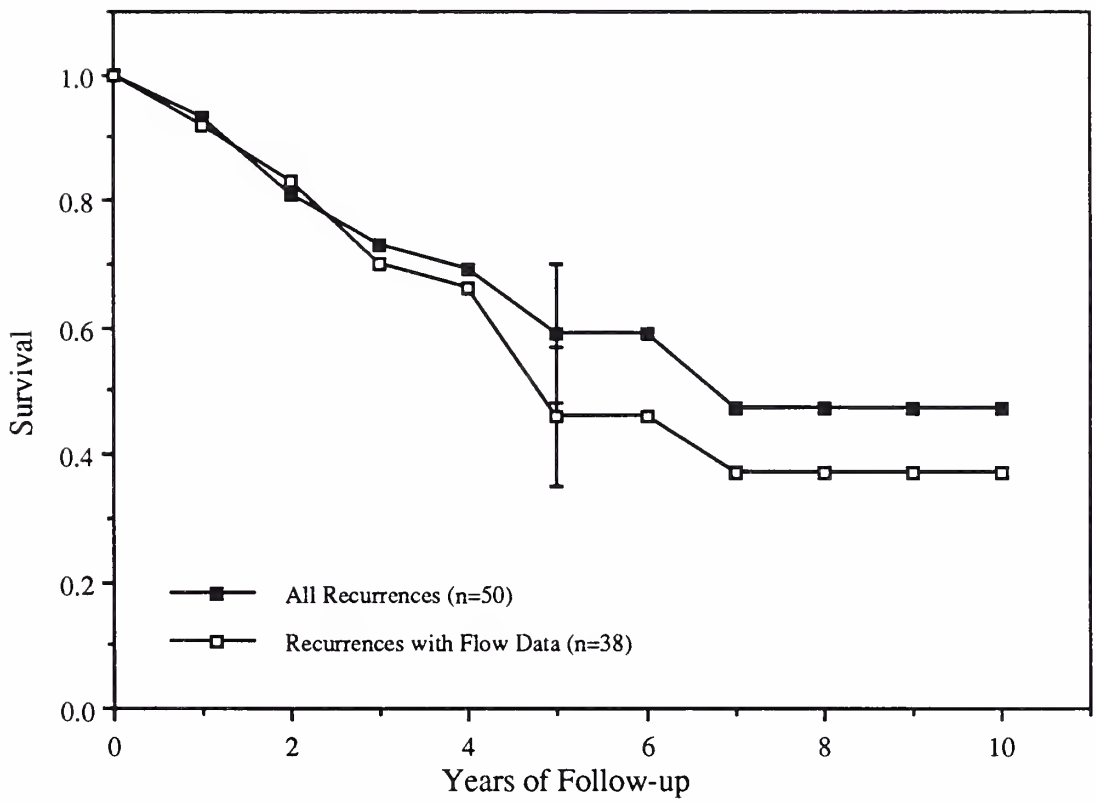


Figure 4

OVERALL SURVIVAL

All Recurrences vs. Recurrences with Flow Data*

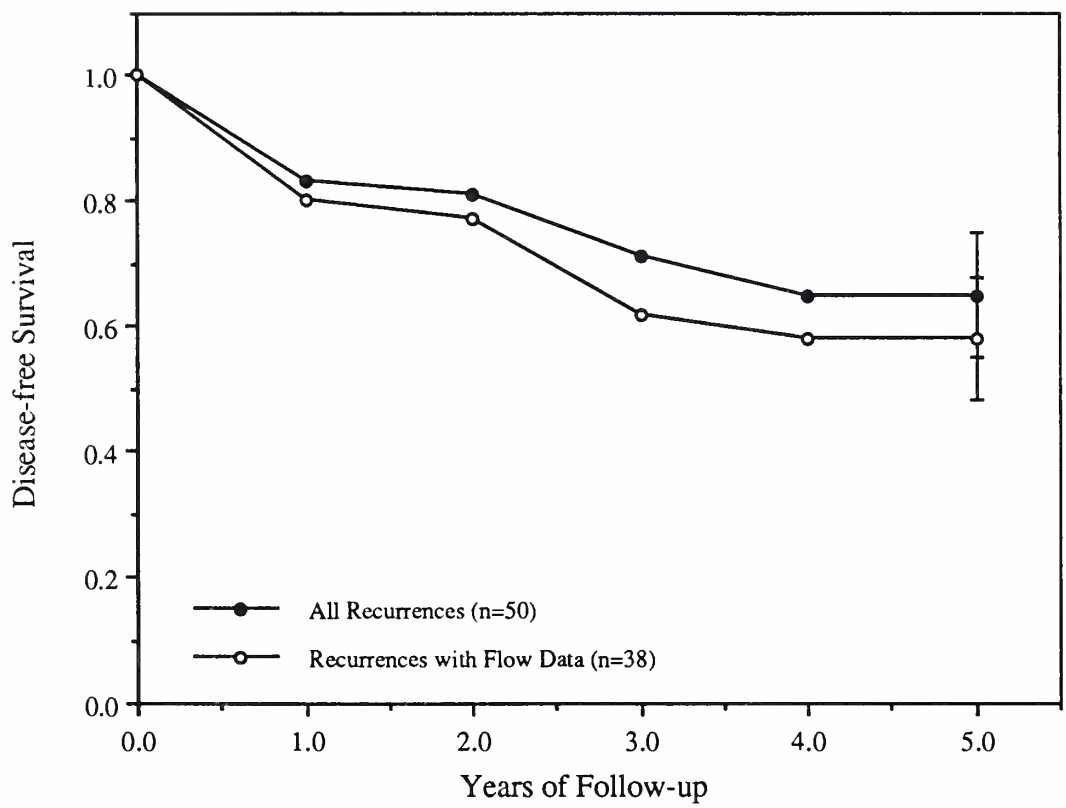


*Actuarial Estimate +/- Standard Error at 5 Years

Figure 5

DISEASE-FREE SURVIVAL

All Recurrences vs. Recurrences with Flow Data*

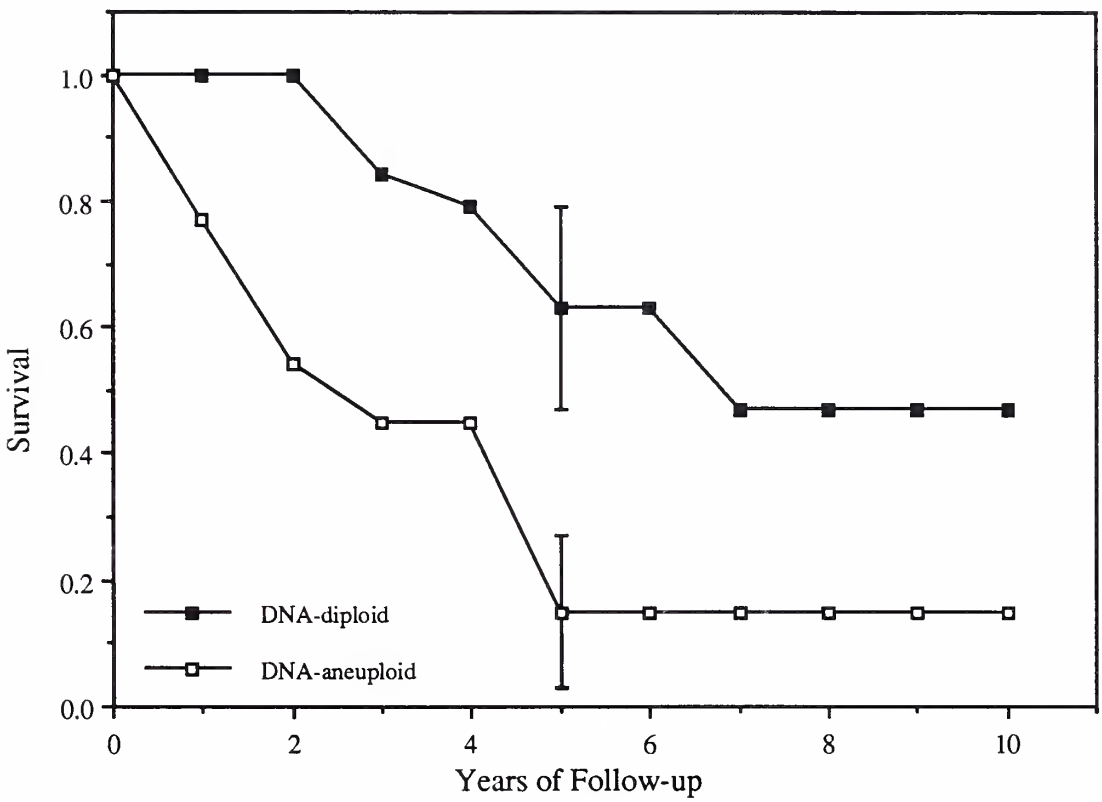


*Actuarial Estimate +/- Standard Error at 5 Years

Figure 6

OVERALL SURVIVAL

By DNA-ploidy at First Breast Recurrence*

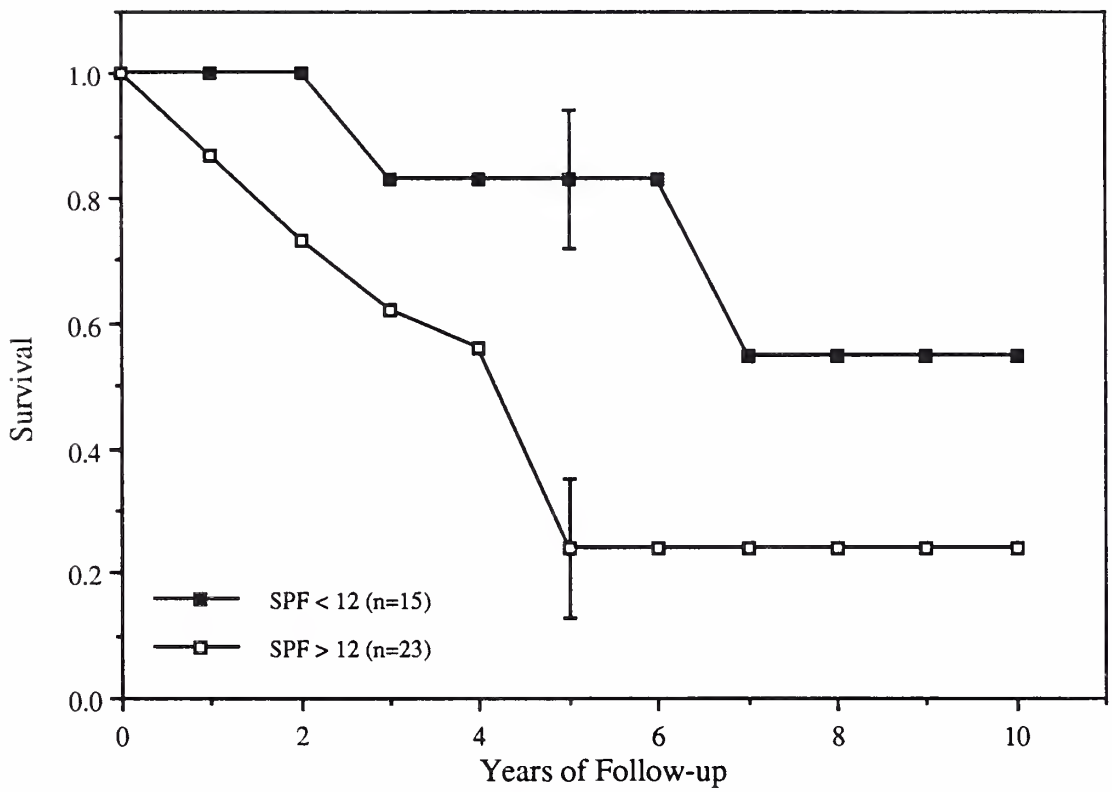


*Actuarial Estimate +/- Standard Error at 5 Years

Figure 7

OVERALL SURVIVAL

By SPF at First Breast Recurrence*

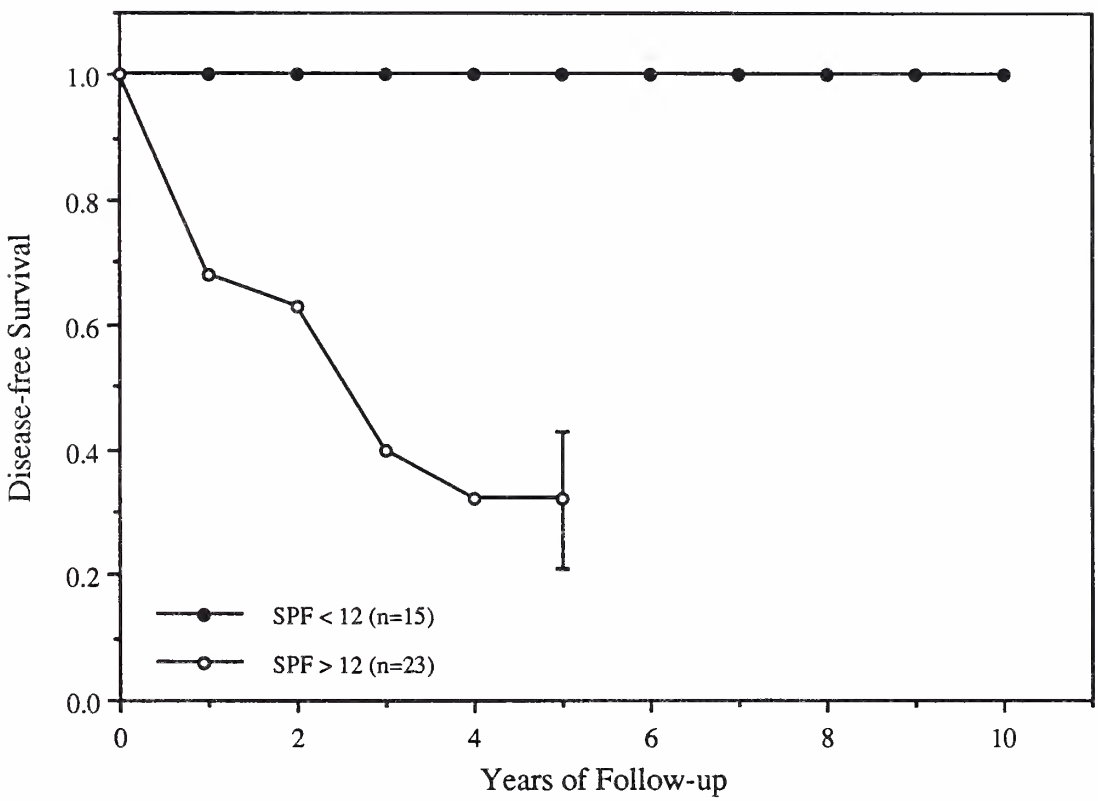


*Actuarial Estimate +/- Standard Error at 5 Years

Figure 8

DISEASE-FREE SURVIVAL

By SPF at First Breast Recurrence*

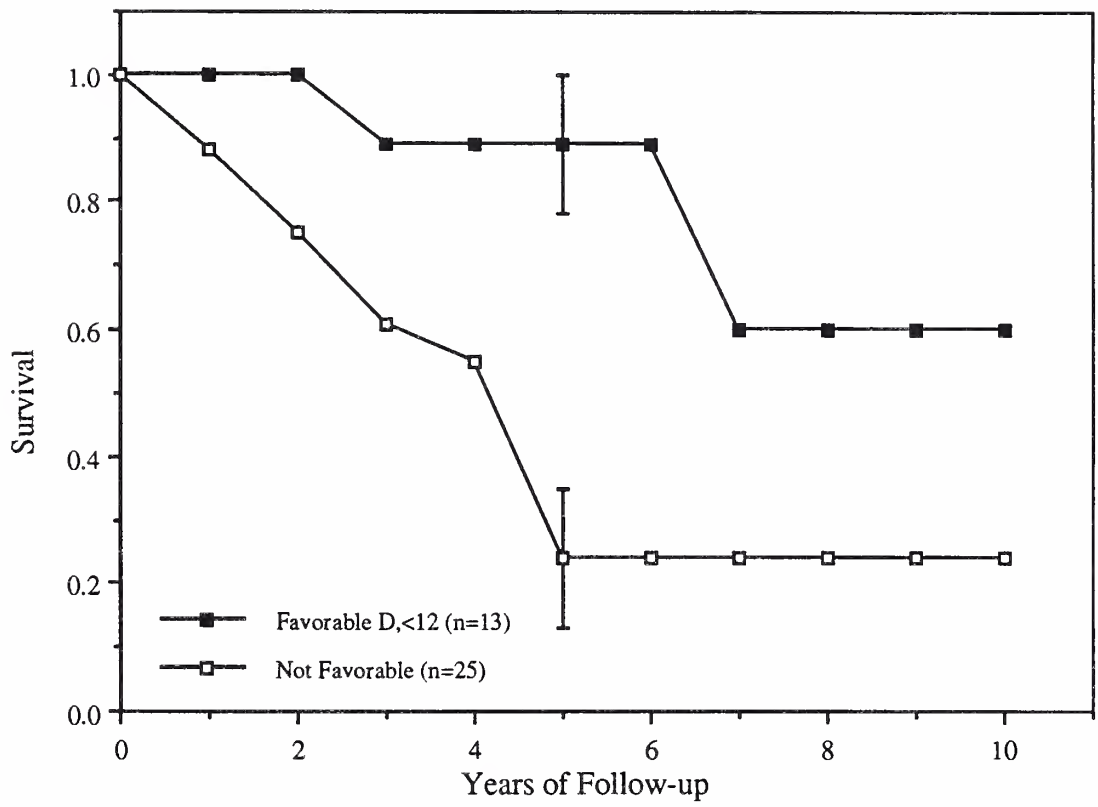


*Actuarial Estimate +/- Standard Error at 5 Years

Figure 9

OVERALL SURVIVAL

By Flow Profile at First Breast Recurrence*

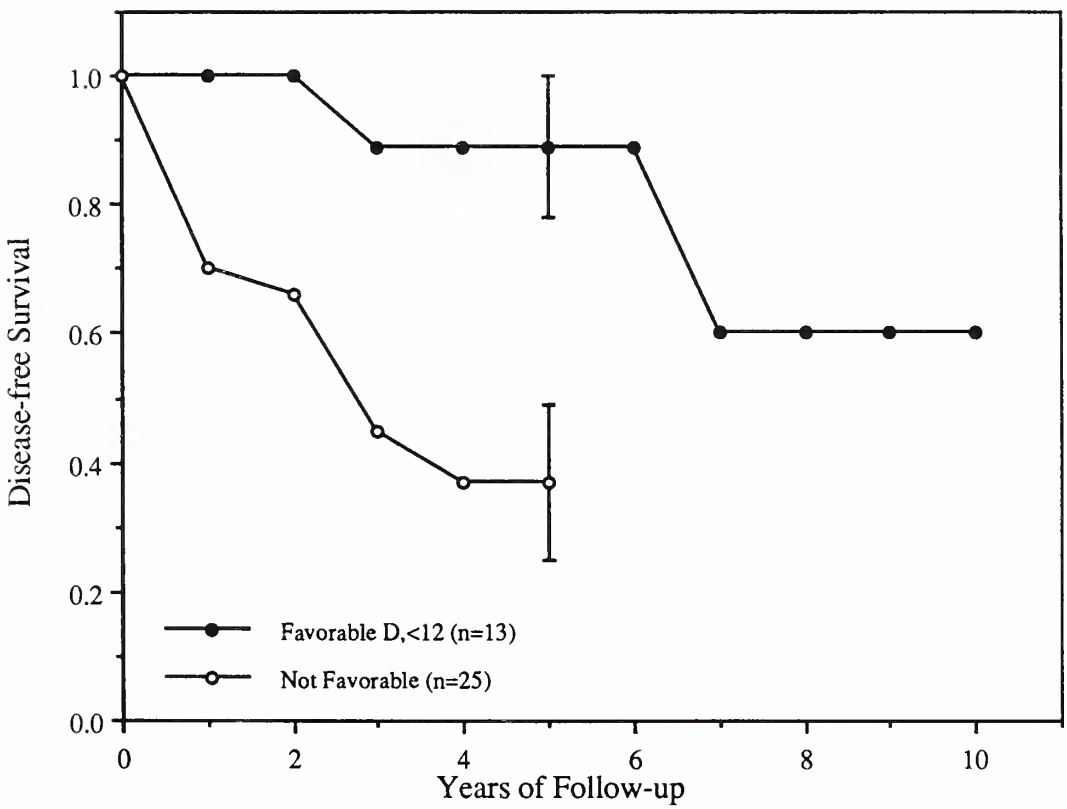


*Actuarial Estimate +/- Standard Error at 5 Years

Figure 10

DISEASE-FREE SURVIVAL

By Flow Profile at First Breast Recurrence*



*Actuarial Estimate +/- Standard Error at 5 Years

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